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Ambiguous Origin: Two Sides of an Ephrin Receptor Tyrosine Kinase

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The assembly of a functional receptor tyrosine kinase via expressed protein ligation using receptor segments produced in two different organisms by Singla et al. (2011) provides a tool for monitoring the order of tyrosine phosphorylation events upon ligand activation.

Expressed protein ligation (EPL) (Muir et al., 1998) is typically used to link synthetic peptides to recombinantly expressed proteins in order to obtain engineered proteins with new properties. However, this approach can also be used to generate hybrid proteins by overexpression in two different organisms as demonstrated by Singla et al. (2011). A variety of reasons can be imagined to adopt such a hybrid synthesis strategy: e.g., the unique abilities of different expression organisms to introduce specific posttranslational modifications such as glycosylation or lipidation, or the need for isotope labeling of protein segments and the functional expression of certain protein domains. The latter case can be extremely challenging when dealing with complicated multidomain proteins from eukaryotic sources that are expressed in bacterial systems such as *E. coli* as nonfunctional polypeptides. Since protein folding of such multidomain proteins remains a challenge that can often not be successfully addressed, solutions are required that allow a functional expression of domains in such multidomain assemblies (Drew et al., 2003). In many cases, suitable redox conditions during expression and purification are essential for obtaining functional proteins. The reducing environment in bacteria can be detrimental to folding of protein domains that relies on the formation of disulfide bonds to stabilize their three-dimensional structure. The use of suitable (eukaryotic) expression systems can overcome this problem. However, in certain cases, proteins contain different parts that require an oxidizing environment or prefer reducing conditions, which makes it difficult to reconcile the requirements for functional expression of both parts in one system.

Such problems are especially noticeable in proteins that reside in different environments separated by a membrane, as in the case of many cell surface receptors. Functional expression of separate domains of these receptors is easily achieved in a variety of expression systems, but the expression of full-length receptors is rather challenging. Expressed protein ligation suggests itself as a solution to link separately expressed receptor domains.

Several pitfalls have to be considered when adopting EPL for the assembly of a full-length receptor molecule containing an oxidized and a reduced part. These are best highlighted in the current example by Singla et al. (2011) that elegantly describes the successful assembly of an Ephrin (Eph) receptor tyrosine kinase by EPL (Figure 1). Eph receptors form the largest family of receptor tyrosine kinases comprising two subclasses: A and B (EphA1-A10 and EphB1-B6). They are involved in cell-cell signaling upon interaction with their membrane-bound ephrin ligands (Lackmann and Boyd, 2008). The EphA4 receptor itself consists of a large extracellular domain (ectodomain) comprising the ephrin-ligand binding domain, a cysteine-rich domain that contains disulfide bridged cysteines and two fibronection-type III domains. This part of the receptor needs to be expressed under oxidizing conditions in order to obtain functional domains, e.g., secreted from insect cells, whereas the cytoplasmic domain can be expressed under reducing conditions as found in bacteria (Figure 1). However, the intein required for generating a C-terminal α-thioester on the extracellular domain contains two internal cysteines not involved in the splicing reaction that appear to be sensitive to oxidizing conditions and therefore were replaced by serine residues to prevent formation of nonnative disulfide bonds (Singla et al., 2008). Furthermore, the C-terminal amino acid that carries the α-thioester after intein cleavage heavily influences cleavage efficiencies; careful mutations can help to increase protein-α-thioester yields dramatically.

The cyctoplasmic part of Eph receptors comprises a juxtamembrane domain that contains tyrosine phosporylation sites, a kinase domain, a sterile-alpha motif interaction domain (SAM), and a PDZ-binding motif. Singla et al. (2011) have expressed cytoplasmic receptor segment comprising the juxtamembrane domain and the kinase domain N-terminally fused to the small ubiqutin-like modifier (SUMO) protein. Upon treatment with the SUMO protease, the cytoplasmic domain is released with an N-terminal cysteine for the EPL reaction with the extracellular domain (Malakhov et al., 2004). This N-terminal cysteine can either be a native residue or needs to be introduced as a nondeleterious mutation.

An additional challenge lies within the conditions for such a ligation reaction between two protein segments that requires the presence of small molecule thiols as ligation mediators and reducing agents (Johnson and Kent, 2006). However, due to sensitivity of the extracellular domain toward reducing environments, careful adjustments are necessary to identify conditions that lead to satisfactory ligation yields without interfering with folding, as well as function of the extracellular domain.

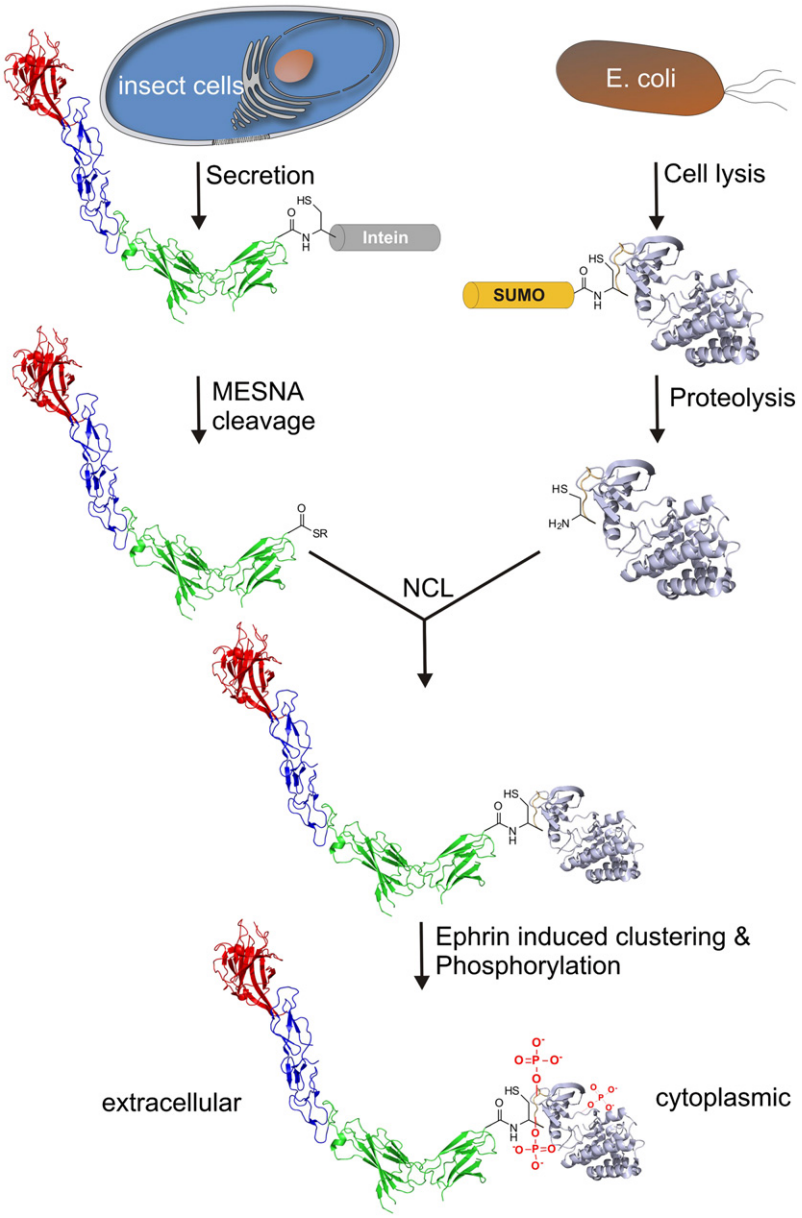

Figure 1. Synthesis of a Hybrid Eph Tyrosine Kinase Receptor by Expressed Protein Ligation
The extracallular domain comprising the ligand-binding domain (red), the cysteine-rich domain (blue), and two fibronection-type III motifs (green; PDB entry 3FL7) is expressed in insect cells fused to an intein and an affinity tag for simple purification (not shown). The cytoplasmic part of the receptor comprising a small stretch of the juxtamembrane domain (yellow) and the kinase domain (cyan; PDB entry 2HEL) is expressed in *E. coli* with an N-terminal SUMO tag. Processing of both constructs provides access to the extracellular domain with a C-terminal thioester group and the cytoplasmic part with an N-terminal cysteine residue for native chemical ligation. The resulting Eph receptor is missing its native transmembrane domain in between the ectodomain and the cytoplasmic part of the receptor, as well as the C-terminal SAM domain and the PDZ-binding motif. Activation of the hybrid receptor with clustered ephrin A leads to autophosphorylation of tyrosine residues first in the juxtamembrane domain (yellow) and subsequently in the activation loop of the kinase domain. The order of phosphorylation is indicated by the different sizes of the phosphate groups.

This synthesis strategy provides a general method for accessing sufficient amounts of functional full-length cell surface receptors for biochemical and structural studies on this still elusive but highly important class of proteins. An advantageous extension of this approach could be based on the use of split inteins that would render the intein as well as the protease cleavage reaction superfluous (Mootz, 2009). Additional benefits would be the use of serine instead of cysteine side chains as nucleophiles during splicing by some split inteins, and that no significant concentrations of thiols as ligation mediators would be required. Problems related to the reduction of native disulfide bonds could therefore be avoided. However, the efficiency of split intein reactions depends on the specific amino-acid context, and optimizing *trans*-splicing reactions can be challenging.

The multimilligram amounts of EphA4 receptor obtained by EPL were analyzed with respect to biological function, especially trans- and autophosphorylation upon ephrin activation. The individual domains within the hybrid receptor retain full activity as demonstrated by substrate ligand activation, substrate binding, and phosphate transfer activity. Autophosphorylation events crucial for regulation of kinase activity were monitored by mass spectrometry and revealed a surprising order of phosphorylation events on the cytoplasmic receptor domain. These events occurred upon ephrin-binding to the receptor and were most pronounced when ephrin was previously prearranged in clusters. A tyrosine residue in the juxtamembrane region is most important for receptor activation, whereas the usual suspect, a tyrosine residue in the activation loop of the kinase domain, is less important. Even for the two tyrosine residues in the juxtamembrane domain, a specific phosphorylation order is observed as indicated in Figure 1 by the differently sized phosphate groups. Other studies have already suggested a key role of this specific juxtamembrane tyrosine residue in Eph receptor signaling supporting the findings of Singla et al. (Shintani et al., 2006). However, questions remain regarding the mechanism of signal transduction from the extracellular ligand-binding domain toward the intracellular kinase domain. These can be addressed in the future with sufficient amounts of receptor made available by the discussed EPL approach. Furthermore, one missing part of the receptor should be included in future studies: the single transmembrane (TM) helix separating extracellular and cytoplasmic parts of the receptor. Advances in handling such hydrophobic sequences in protein synthesis and semisynthesis approaches should allow the synthesis of receptors including only

one such membrane-spanning domain (Olschewski and Becker, 2008).

The approach by Singla et al. (2011) will hopefully trigger the synthesis of many more full-length cell surface receptors, as they are a highly important class of proteins in signal transduction and as drug targets.

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Elegant Metabolite Biosynthesis

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Hormaomycin, an NRPS-produced bacterial metabolite involved in microbial signaling, possesses several remarkable structural features. The study by Höfer et al. (2011) employed a range of methodologies to explore and ultimately understand the elaborate biosynthesis of this complex natural product.

Bacteria interact and communicate with other bacteria in a multitude of ways. For example, quorum sensing is used to control density-specific phenotypes that, in turn, enable behaviors such as biofilm formation, virulence, or production of antibiotics and other secondary metabolites. Quite often, small autoinducer molecules such as homoserine lactones, furanosyl borate diesters, and oligopeptides are employed to direct these behaviors by regulating gene expression (Ng and Bassler, 2009) and, in the case of antibiotic production, to activate biosynthetic gene clusters encoding the production of additional metabolites that provide defense for bacteria (El-Sayed et al., 2001). The effectiveness of these latter compounds is in part due to their chemical diversity, as incorporation of unusual functional groups or elements such as halogen atoms can increase both their specificity and biological activity. Recently, it has become possible to directly visualize these types of chemical interactions between bacteria using MALDI imaging mass spectrometry (Yang et al., 2009).

In a remarkable paper published in this issue of *Chemistry and Biology*, Höfer et al. (2011) characterize the biosynthesis of hormaomycin, a highly unusual nonribosomal peptide synthetase (NRPS)-derived molecule from *Streptomyces griseus*. Hormaomycin (also known as takaokamycin) (Omura et al., 1984) induces morphological differentiation and secondary metabolite production in actinobacteria, and is also a potent narrow spectrum antibiotic (MIC 88 pM against coryneform actinobacteria). Thus, hormaomycin is a rare example of a natural product that can both regulate bacterial behaviors and also independently function in a defensive antibiotic role.

Hormaomycin is composed of several unusual residues, making it quite possibly the most structurally unique microbial morphogen yet described. Most dramatically, it possesses two alanine residues with nitro-cyclopropyl groups [(3-Ncp)Ala], two β-methyl phenylalanine residues [(β-Me)Phe], a novel chlorinated pyrrole [5-chloropyrrole 2-carboxylic acid (Chpca)], and a proline unit with a 4-propenyl substituent [4-((Z)-propenyl) proline (4(-Pe)Pro)] (Figure 1). Although the structure of hormaomycin has been confirmed via total synthesis, insights into its biosynthesis have until now been limited to feeding experiments focused on the two unusual (3-Ncp)Ala residues and have revealed their ultimate origin from L-lysine (Brandl et al., 2005). Additional prior work revealed that alternate precursor amino acids could be incorporated into this unit, producing a hormaomycin analog with altered biological properties (activity against the fungus *Candida albicans*) (Zlatopolskiy et al., 2006). One other study identified the pyrrole halogenase from this gene cluster (ultimately HrmQ) and demonstrated that it could be inserted into the chlorobiocin biosynthetic pathway in *Streptomyces roseochromogenes* var. *oscitans*, thereby producing two novel analogs (Heide et al., 2008).